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Potentiation of Antitumor Activity of Mitomycin C by Estradiol: Studies of Human Breast Carcinoma Xenografts Serially Transplanted Into Nude Mice

SHOICHI OKA, MD, TETSURO KUBOTA, MD, TOORU TAKEUCHI, MD, AND MASAKI KITAJIMA, MD, FACS
From the Department of Surgery, School of Medicine, Keio University, Tokyo, Japan

The effect of experimental cancer chemotherapy with mitomycin C (MMC) was studied using three estrogen-receptor (ER)-positive (MCF-7, R-27, and Br-10) and one ER-negative (MX-1) human breast carcinoma xenograft serially transplanted into nude mice, and the effect of estradiol (E2) priming on the antitumor activity of MMC was investigated. Intramuscular injection of E2 at 1 mg/kg changed the ER state and increased the growth fraction detected by flow cytometry, although the growth rate of ER-positive tumors was not effective by E2 priming. MMC suppressed the growth of the four xenografts in a dose-dependent manner. When 1 mg/kg E2 was administered 1 h before MMC treatment, which was given intraperitoneally at a dose of 3 mg/kg, the antitumor activity of MMC was increased in comparison with MMC alone in ER-positive strains, although the effect of MMC on MX-1 was not changed by E2-priming. Priming with E2 at this dose increases the growth fractions of ER-positive breast carcinoma cells, which are sensitive to MMC, resulting in increased antitumor activity of MMC. This E2-primed MMC chemotherapy may be of value in the treatment of ER-positive human breast cancer.

KEY WORDS: **estradiol, mitomycin C, growth fraction, experimental cancer chemotherapy, breast cancer**

INTRODUCTION

In spite of improved cure rates for early breast cancer, the treatment of advanced breast cancer still remains unsatisfactory. The 10-year survival rates for stage III and IV cancers in our department are 28% and 0%, respectively [1]. Although various kinds of adjuvant therapy have been used for advanced breast cancer, conventional antitumor agents give a response rate of only 30–40% [2,3]. Breast carcinoma originates from glandular tissue, which is regulated by estradiol, and 30% of breast cancers as a whole and 60% of estrogen receptor (ER)-positive breast cancers are regulated by estradiol and sensitive to endocrine therapy [4]. It is also reported that tumor cells in the growth fraction (GF) are more sensitive to chemotherapeutic agents in both rodents [5] and humans [6], i.e., rapidly growing tumors with a high GF are more sensitive to antitumor agents.

Some researchers have attempted to modulate the chemosensitivity of MCF-7, a cultured breast cancer cell line, by increasing the GF using estradiol in vitro, and a positive effect of this combination has been reported in some cases [7–9]. However, since E2 can potentially increase the growth of breast cancer, resulting in more rapidly progressive disease, this combination antitumor treatment should be evaluated using an in vivo system

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Address reprint requests to Tetsuro Kubota, MD, Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

to determine the optimal treatment schedule including the dose and timing of estradiol administration. We have conducted experimental chemotherapy using a combination of E2 and mitomycin C (MMC) on three ER-positive and one ER-negative human breast carcinoma xenograft serially transplanted in nude mice in order to find the optimum conditions for E2 priming to increase the antitumor activity of MMC without enhancing tumor growth.

MATERIALS AND METHODS

Nude Mice

Female nude mice with a BALB/cA genetic background were purchased from CLEA Japan Co. (Tokyo). They were maintained under specific pathogen-free conditions using an Isorack in our experimental animal center and fed sterile food and water ad libitum. Mice, 6–8 weeks old, weighing 20–22 g were used for the experiments.

Human Tumor Xenografts

Three ER-positive human breast carcinoma xenografts, MCF-7, R-27, and Br-10, and one ER-negative strain, MX-1, were used for the experiments. MCF-7 was established as a cultured cell line by Soule et al. [10] in 1970 and was successfully transplanted into nude mice treated with estrogen and progesterone in our department in 1983 [11]. R-27 was transplanted from a tamoxifen-resistant variant of MCF-7 [12] by the same procedure. Br-10 was established from free-floating cancer cells in pleural effusion from a 43-year-old woman with invasive ductal carcinoma in 1974 by Hirohashi et al. [13] and was kindly supplied to our institute by Dr. Hirohashi. MX-1 was established by Giovanella et al. [14] from breast cancer tissue of a 29-year-old female patient and was kindly supplied to our institute by Dr. K. Inoue (Cancer Chemotherapy Center, Tokyo), in 1979.

Agents

On the day of tumor inoculation, 5 mg of 17beta-estradiol dipropionate and 250 mg of 17alpha-progesterone caproate (Pg), administered as 0.1 ml of EP hormone depot^R (Teikoku Zoki Co., Tokyo) were given intramuscularly to allow exponential tumor growth of ER-positive strains.

Treatment was initiated when the tumors reached 100–300 mg >2 weeks after tumor inoculation, by which time the serum estradiol level had decreased to that of untreated female mice [15]. Commercially available mitomycin C (MMC; Kyowa Hakko Kogyo Co. Tokyo) and 17beta-estradiol dipropionate (E2; Ovahormone Depot^R; Teikoku Zoki) were used. E2 at 1 mg/kg was administered intramuscularly into the thigh using a 26-gauge tuberculin needle. MMC was administered intraperitoneally as a single dose of 1, 2, 3, or 4.5 mg/kg dissolved in 0.2 ml of saline using a 26-gauge tuberculin needle. The antitumor activity of 3 mg/kg MMC was also evaluated using a dosing schedule of q4d × 3. In studies of combined therapy, MMC was administered at 3 mg/kg 1 h after treatment with E2 at a dose of 1 mg/kg, and the effect of the combination was compared with that of 3 mg/kg MMC alone.

Tumor Inoculation, Measurement of Tumor Size, and Evaluation of Agent Activity

Two fragments of tumor tissue, each measuring 3 × 3 × 3 mm, were inoculated into the subcutaneous tissue of the bilateral dorsum of ether-anesthetized nude mice, using a trocar needle. The tumors were measured by length and width, using sliding calipers, three times weekly by the same observer.

Tumor weight was calculated from linear measurements using the formula of Geran et al. [16]: tumor weight (mg) = length (mm) × [width(mm)]$^2/_2$. When the tumors reached 100–300 mg, the tumor-bearing mice were allocated randomly to test groups each consisting of between 4 and 6 animals. The tumor growth curve of the estimated tumor weight against time after initial treatment was plotted. Tumor doubling time (Td) was calculated using the formula; $Td = \log 2/b$ and $\log(Wi) = a + b \times (Day)$, where Wi is the mean tumor weight at any given time and Day is the number of days after tumor inoculation [17]. Td was calculated for each tumor, and the mean and standard deviation of Td were calculated for each group.

The relative mean tumor weight (RW) was calculated as RW = Wi/Wo, where Wi is the mean tumor weight at any given time and Wo is the mean tumor weight at the time of initial treatment. The antitumor effects of the agents were evaluated in terms of the lowest T/C value (%) during the experiment, where T is the relative mean tumor weight of the treated group and C the relative mean tumor weight of the control group at any given time. The antitumor activity was evaluated as positive when the lowest T/C was <=42%.

The dose-response antitumor activity of MMC was calculated using the linear regression equation; $\log(T/C) = a - b \times (Dose)$, where T/C is the lowest T/C of the relative mean tumor weight and Dose is the administered dose of MMC. The reliability of this equation was confirmed by *t*-test of the correlation coefficient between log(T/C) and Dose. To assess the enhancement of MMC antitumor activity by E2, the value of T/C for E2 + MMC was replaced into the regression for MMC alone and the corresponding single dose of MMC giving the same antitumor activity was calculated.

Hormone Receptor Assay

Estrogen receptor (ER) and progesterone receptor (PgR) were assessed by the dextran-coated charcoal method and exchange assay in each tumor. Transplanted

TABLE I. Effects of Estradiol and Mitomycin C on Growth of Human Breast Carcinoma Xenografts

Drug	Dose (mg/kg)	MCF-7	R-27	Br-10	MX-1
		Tumor doubling time in days (M ± SD)[a]			
Estradiol	0	15.9 ± 5.8	15.9 ± 4.4	10.8 ± 4.4	6.4 ± 1.1
	1	14.1 ± 5.2	15.3 ± 3.3	7.4 ± 1.0	6.6 ± 0.9
		Lowest T/C ratio of relative mean tumor weight[b]			
Mitomycin C	0	100	100	100	100
	1	96.6	91.1	69.3	49.2
	2	85.5	88.8	55.1	35.9
	3	72.0	87.2	53.0	25.2
	4.5	79.2	51.2	40.8	ND
	9	23.4	11.7	22.4	7.9

[a]Tumor doubling time (Td) was calculated using the formula: Td = log2/b and log(Wi) = a + b × (Day), where Wi is the mean tumor weight at any given time and Day is the number of days after tumor inoculation.
[b]The lowest T/C value (%) during the experiment was calculated, where T is the relative mean tumor weight of the treated group and C the relative mean tumor weight of the control group at any given time.
ND = not done.

tumors were resected at 12, 24, 36, 48, and 72 h after injection of 1 mg/kg E2, and then stored at −80°C for detection of ER and PgR using the method reported previously [18,19]. Positive criteria for ER and PgR included a binding site concentration of 10 fmol/mg protein and a dissociation constant (Kd) of 10^{-10}M.

Flow Cytometric Analysis

Flow cytometric analysis using the method of Nakamura [20] with an EPICS-V flow cytometer. The mechanically dissociated tumor cells were fixed with 99.99% ethanol, the cytosol fractions were digested with RNase and Triton-X, and DNA of the remnant tumor cells was stained with propidium iodide. After the DNA pattern had been detected, the DNA histogram was analyzed using the method of Bagwell et al. [21], and the S-phase fraction (%S) calculated. S-phase fractions were determined in the tumors 12, 24, 36, 48, and 72 h after administration of E2 at a dose of 1 mg/kg.

RESULTS

The effects of E2 and MMC alone against the four human breast carcinoma xenografts are shown in Table I in terms of Td for E2 and the lowest T/C for MMC. Tds ranged from 6.4 to 15.9 days, and these values were not shortened significantly by the administration of E2, where *t*-values were 0.566 for MCF-7, 0.267 for R-27, 1.846 for Br-10, and 0.345 for MX-1, demonstrating that E2 at a dose of 1 mg/kg did not enhance tumor growth. The antitumor effect of MMC on the xenografts was dose-dependent and the minimum effective dose of MMC was 9 mg/kg for MCF-7 and R-27, 4.5 mg/kg for Br-10, and 2 mg/kg for MX-1 (Table I). The dose-dependence was statistically significant with linear regression coefficients of correlation of 0.961 for MCF-7, 0.950 for R-27, 0.981 for Br-10, and 0.965 for MX-1 (all $P < 0.05$) (Table II).

The antitumor activity of MMC at 3 mg/kg on Br-10 with or without E2 priming is shown in Figure 1. The positive antitumor activity of MMC alone was significantly enhanced by E2 priming, although E2 alone did not inhibit the growth of Br-10 (Table I). The effects of MMC with or without E2 are summarized in Table II, in which the T/C with E2 + MMC and the regression equation for MMC alone are shown. T/Cs with E2 + MMC were 46.1%, 59.4%, and 27.0% for MCF-7, R-27 and Br-10, respectively, which were lower than those with MMC alone in each case. When the values of T/C for E2 + MMC were substituted into the regression equation for the dose-dependent activity of MMC alone, the equi-active doses of MMC were calculated to be 5.8, 3.2, and 7.4 mg/kg for MCF-7, R-27 and Br-10, respectively. However, the antitumor activity of MMC against ER-negative MX-1 was not increased by E2-priming.

Table III shows the changes in the S-phase fraction and binding sites of hormone receptors in xenografts treated with E2 at a dose of 1 mg/kg. ER-negative MX-1 was excluded from this experiment. The control S-phase fraction of Br-10 was 24.5%, which was higher than those of MCF-7 and R-27 (14.7% and 22.8%, respectively). The results for growth rate were comparable in that the Td value for Br-10 (10.8) was higher than those for the other xenografts. The S-phase fraction was increased by E2 administration, peaks occurring at 12, 72, and 24 h in MCF-7, R-27 and Br-10, respectively. In all the control xenografts, ER and PgR were positive with binding sites concentrations of >10 fmol/mg protein and dissociation constants of 10^{-10}M (data not shown). The

TABLE II. Effects of Mitomycin C With and Without Estradiol Pretreatment Against Human Breast Carcinoma Xenografts Serially Grown in Nude Mice

Tumor	E2 + MMC[a]	MMC[b]	Regression equation[c]	r[d]	Dose[e]
MCF-7	46.1[f]	72.3	logT/C = 2.066 − 0.069 × Dose	0.961	5.8
R-27	59.4	87.2	logT/C = 2.108 − 0.110 × Dose	0.950	3.2
Br-10	27.0	53.0	logT/C = 1.943 − 0.070 × Dose	0.981	7.4
MX-1	34.5	25.2	logT/C = 1.842 − 0.111 × Dose	0.965	2.7

[a] 17beta-estradiol dipropionate (E2) was preadministered intramuscularly at a dose of 1 mg/kg 24 h before intraperitoneal administration of mitomycin C (MMC) at a dose of 3 mg/kg.
[b] Mitomycin C (MMC) was administered alone at 3 mg/kg.
[c] Regression equation of antitumor activity of MMC in T/C (treated/control) and dose of MMC (mg/kg).
[d] Coefficient of correlation (r) between antitumor activity of MMC in T/C and dose of MMC (mg/kg).
[e] Equi-active dose of MMC in mg/kg.
[f] Lowest T/C value for relative mean tumor weight.

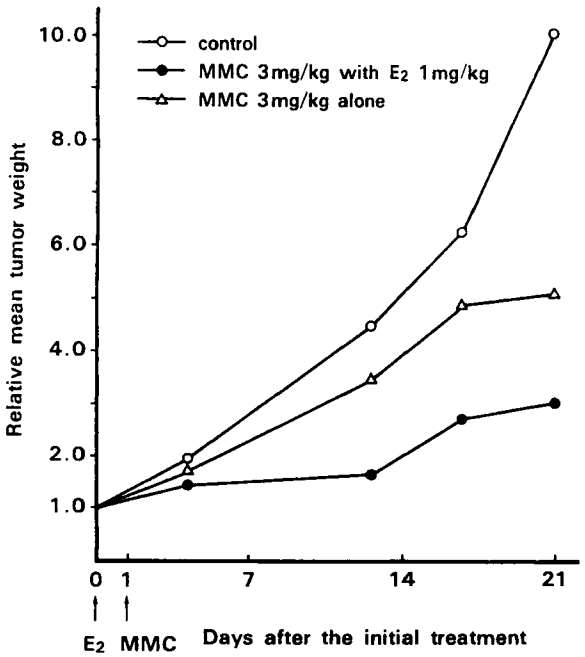

Fig. 1. Antitumor activity of mitomycin C on Br-10, a human breast carcinoma xenograft grown serially in nude mice with or without estradiol priming. Mitomycin C (MMC) at 3 mg/kg was administered 1 h after treatment with 17beta-estradiol dipropionate (E2) at a dose of 1 mg/kg, and the effect of the combination was compared with that of MMC alone at 3 mg/kg. The positive antitumor activity of MMC alone was significantly enhanced by E2 priming.

TABLE III. Effects of Estradiol on Percentage S-Phase Fraction and Hormone Receptors of Human Breast Carcinoma Xenografts Serially Grown in Nude Mice

	Time[a]	MCF-7	R-27	Br-10
S fraction[b]	0	14.7	22.8	24.5
	12	19.1	24.3	29.2
	24	17.0	28.0	29.8
	36	7.9	21.8	17.2
	48	ND	26.8	21.9
	72	ND	31.9	28.7
ER[c]	0	68	10	50
	12	39	11	105
	24	38	12	18
	36	30	11	8
	48	20	11	11
	72	ND	12	ND
PgR[d]	0	58	34	120
	12	20	33	80
	24	18	31	70
	36	35	20	4
	48	25	30	3
	72	ND	36	ND

[a] Hours after estradiol treatment at a dose of 1 mg/kg.
[b] Detected by flow cytometry expressed as a percentage.
[c] Cytosol estrogen receptor concentration in fmol/mg protein.
[d] Cytosol progesterone receptor concentration in fmol/mg protein.
ND = not done.

changes in hormone receptors with time varied among the three xenografts. In MCF-7, ER was decreased at 12 h and did not recover within 48 h after E2 priming, whereas PgR decreased from 12 h after E2 treatment. No significant changes in hormone receptors were observed in R-27. ER in Br-10 increased transiently at 12 h after E2 administration and decreased from 24 h, whereas PgR was decreased 12 h after E2 treatment.

DISCUSSION

Although surgery is still the mainstay of treatment for breast cancer, its role is limited in the management of stages III and IV disease, which are associated with low survival rates [1]. Although chemotherapy increases the survival rate after surgery for advanced breast cancer, the rate of response to conventionally available chemotherapeutic agents is only 30–40%. Agents currently in use include mitomycin C, 5-fluorouracil, doxorubicin, and cyclophosphamide [2,3]. Used in combination, these agents can increase the response rate to 50–60% [22], and recently developed taxanes are reported to give a 40–50% response rate when administered alone as a single dose [23]. However, these high efficacy rates are usually associated with a high incidence of side effects, and chemotherapy does not necessarily result in prolonged survival [24]. Mitomycin C, one of the first-line

drugs in breast cancer, is thought to be effective on tumor cells in the growth fraction. Ohara et al. [25] reported that the sensitivity of HeLa cells to MMC was highest in the G1 and G2 phases and low in the late S2 phase. Barlogie and Drewinko [26] reported that LoVo cells showed the greatest sensitivity to MMC in the G2 phase, followed by the S and G1 phases. We have reported previously that the efficacy of MMC is related to the percentage of growth fraction in human tumor xenografts in nude mice [27], and it is generally accepted that MMC is more effective against tumor cells in the growth fraction than in the G0 fraction. The present study focused on the modulation of MMC activity against breast carcinoma xenografts using E2, which affects the growth fraction of breast cancer cells.

Although estrogen is a promoter of ER-positive breast cancer, it was widely-used as an anti-breast cancer agent with a response rate of >20% [28] until the development of the anti-estrogen, tamoxifen, in the early 1970s. In fact, in an early comparative clinical trial [29], the efficacy rate of tamoxifen was not superior to that of diethylstilbestrol. However, the significantly reduced incidence of side effects with tamoxifen encouraged its widespread clinical use. This apparently contradictory action of estrogen in both promoting and suppressing breast cancer has been partly explained by the study by Horwitz and McGuire [30]. They reported that serum 17beta-estradiol enters the target cells by diffusion and combines with nuclear ER to form E2-ER complexes that accumulate in the nucleus and combine with chromatin acceptor sites, which then promote protein synthesis via mRNA. Massive administration of E2 disrupts the replenishment of the E2-ER complex, and this would be one of the mechanisms of E2 antitumor activity [31].

The present study investigated the modulating effect of E2 on MMC antitumor activity by increasing the growth fraction of cells sensitive to MMC. Since this modulating effect of E2 is observed at low doses that do not elicit the antitumor activity of high-dose E2 therapy, it is important to determine the optimum dose of E2 that does not promote tumor growth but increases growth fraction sensitive to MMC. Our preliminary experiment showed that E2 at a dose of 5 mg/kg reduced the antitumor activity of MMC at 3 mg/kg on Br-10, whereas this combination also suppressed the growth of Br-10 in comparison with controls (data not shown). In the present study, cell kinetic analysis showed an increased S-phase fraction in the three xenografts, suggesting that this dose of E2 can increase the growth fraction of breast cancer cells. However, E2 at a dose of 1 mg/kg did not promote the growth rates of xenografts in terms of doubling time, suggesting that this dose is not effective for reducing the antitumor activity of MMC. Recently, we have also reported that MCF-7 tumor in the "non-growing phase" synthesizes DNA and replicates in nude mice without estradiol supplementation as detected by autoradiography using [^{3}H]-thymidine-uptake labeling index, flow cytometric analysis of bromodeoxyuridine labeling, and mitotic index. Since these tumors are apparently "dormant," the synthesis of DNA and replication of the cells seem to be balanced by tumor cell loss [32]. The long latent period of breast cancer might be partly explained by this "proliferating but non-growing phase" of the cancer cells. The present observation that the S-phase of tumor xenografts was increased without an increase in growth rate might reflect our previous data for MCF-7 in the "dormant" phase.

From our present data, we conclude that the optimum dose of E2 for modulating the antitumor activity of MMC is 1 mg/kg. When the equi-active doses of MMC were calculated according to the regression equation for MMC dose dependency, the modulating effect of MMC was 1.9X for MCF-7, 1.1X for R-27, and 2.5X for Br-10. Since the line of dose-dependent equation was steep for R-27, the modulating effect against MMC was only 1.1X, although the T/C of E2 + MMC on R-27 was 59.4%, 30% higher than that of MMC alone, demonstrating a clear effect of E2 on MMC against R-27. Otherwise, no increased antitumor activity was observed for MMC with E2 priming against ER-negative MX-1.

The present results indicate that priming with an optimum dose of E2 modulates the antitumor activity of MMC against ER-positive breast cancer by increasing the growth fraction sensitive to MMC. Because this modulation occurs only in ER-positive cells, the side effects of MMC will not be enhanced in ER-negative bone marrow cells, one of the major sites of MMC dose-limiting toxicity. Some clinical trials has demonstrated unfavorable effects of hormonal "priming" therapy [33–38]. Before the clinical application of this type of modulation, a comparative pharmacokinetic study of E2 and MMC in both mouse and human and cell kinetic analysis of human breast carcinoma cells will be necessary in order to determine the optimum dose of E2 that will increase the antitumor activity of MMC in a clinical study.

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